INFLUENCE OF *in vitro* AND *ex vitro* PROPAGATION ON THE GROWTH AND DEVELOPMENT OF LINGONBERRY

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Influence of *in vitro* and *ex vitro* Propagation on the Growth and Development of Lingonberry

By

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ABSTRACT

The growth and development as well as the chemical composition of lingonberry (*Vaccinium vitis-idaea* L. ssp. *vitis-idaea*) cultivars 'Splendor' and 'Erntedank' were studied. The cultivars were propagated using conventional softwood cuttings *ex vitro* (SC) or by *in vitro* adventitious shoot regeneration from excised leaves of micropropagated shoots (TC). Morphological data, which includes stem number, branch number, leaf number and fruit yield, diameter and weight, were collected after four growth seasons. Chemical analyses for leaf chlorophyll content and for anthocyanin content and antioxidant activity of the fruit were performed.

The results of this study indicate that TC plants exhibited significantly more vegetative growth than SC plants thus making TC plants ideal candidates for rapid establishment in the field. While the fruits of SC plants had higher levels of anthocyanins as well as increased production of larger fruit, the TC fruits showed a greater antioxidant activity to compliment its superior growth and development. 'Erntedank' out-yielded 'Splendor' in berry production although the larger fruit were produced by 'Splendor'.

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ABBREVIATIONS

ABTS	2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)
ANOVA	Analysis of variance
ARP	Anti-radical power
BM	Basal media
CATMOD	Categorical data modelling
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
LC / MSD	Liquid chromatography / mass spec detector
Min	Minute
nm	Nanometre
PPF	Photosynthetic photon flux
rpm	Revolutions per minute
RAPD	Random amplified polymorphic DNA
RH	Relative humidity
SAS	Statistical analysis software
SC	Softwood stem cutting
TE	Trolox equivalent
TC	Tissue culture
UV / VIS	Ultraviolet / visible light

v / v	volume / volume

"Agriculture is our wisest pursuit, because it will in the end contribute most to real wealth, good morals, and happiness."

Letter from Thomas Jefferson to George Washington (1787)

CHAPTER 1: INTRODUCTION

1.1 Biology and Ecology of Lingonberry

The lingonberry (*Vaccinium vitis-idaea* L.) is a very important indigenous small fruit plant in the Newfoundland and Labrador ecosystem. Lingonberry is known as partridgeberry in Newfoundland or redberry in Labrador (Vander Kloet 1988, Estabrooks 1997). There are more than 25 names for lingonberry depending on regional nomenclature; such as, alpine cranberry, northern mountain cranberry, moss cranberry, foxberry, lingberry, lingen, kokemono, preiselbeeren, puolokka and keepmingyuk (Burt and Penhallegon 2003). The plant is a rhizomatous, low-growing dwarf shrub (Luby *et al.* 1991) (Figure 1.1). The leaves are simple, petiolate, evergreen, leathery and alternate. The upper surface of the leaves are dark green while the lower surface is pale green and waxy with black glandular dots (Camp 1945). The flowers are hermaphroditic, bell-shaped and are commonly pink, although sometimes white in colour (Penhallegon 2006). Plants produce small, dark red berries up to 1.2 cm in diameter (Figure 1.2); the fruit are rich in anthocyanins, antioxidants and flavonoids (Wang *et al.* 2005). There are two major periods of flowering in many European lingonberry varieties, March to April and July to August. The resulting fruit ripen and are harvested respectively in mid-August and mid-October (Penhallegon 2006). In native North American lingonberry there is one flowering period in June or July, resulting in ripe fruit once per season ready for harvest by September (Hillier 2001).

Figure 1.1: The two *V. vitis-idaea* L. subspecies, left: ssp. *vitis-idaea*; right: ssp. *minus* (Pot diameter: 12 inches).

However there is seasonal variation in ripening time depending on weather and plant health. The fruit can be consumed raw or used in wines, pastries, baked goods, jams and jellies (Launert 1981).

The lingonberry has distinctive morphological characteristics that make it a promising candidate for intensive agricultural development in Newfoundland and Labrador's ecosystems; it is cold hardy and can withstand windy environments (Jamieson 2001). It is deep rooting and has rapidly wide-spreading rhizomes that spread up to 200 m per m^2 (Tear 1972). This rhizome network maximizes surface area for nutrient and water absorption and also helps prevent frost heave, a common problem in Newfoundland and Labrador. The dense rhizome network produces closely spaced plants that can yield up to 500 flowers per m^2 (Tear 1972).

Lingonberry plants grow in large naturally-occurring stands and although these appear to be homogeneous there is a large amount of genetic diversity therein (Gustavsson 2001). The lingonberry reproduces sexually by fruit production or asexually by vegetative regeneration from rhizomes. Pollination of lingonberry plants occurs mainly via insect pollinators. The most common pollinators of lingonberry flowers are bumble bees (*Bombus* spp.) which are thought to be attracted to the plants by the vibrant colour and aromatic scent of the flowers (Davis *et al.* 2003). Although plants are self-compatible, previous studies suggest that cross-pollination produces larger fruit and increased fruit yield (Gough 1994). The plant will produce few, if any flowers, in the wild until the plant is five years old (Burt and Penhallegon 2003). Each fruit contains between three and fifteen seeds and are either dropped to the ground after ripening or

dispersed by animal vector, typically birds and mammals (Nuortila *et al.* 2002). Seed dispersal is followed by germination if favourable characters such as soil pH, moisture levels, photoperiod and temperature are met. However, it is uncommon to see seedlings in the wild as the majority of lingonberry reproduction is done by rhizomatous shoot production (Gustavsson 2000).

Increase in plant numbers is accomplished by means of vegetative shoot production from laterally spreading rhizomes. The plant produces rhizomes which are creeping and can grow through peat soil and mineral soil. New auxiliary shoots develop at the nodes of these rhizomes; if separated from the original plant these would develop into new plantlets (Tear 1972). The axillary stems are produced using the same developmental processes as branch production. These rhizomatous shoots develop into a mature adult plant and begin developing its own root system.

1.2 Taxonomy

The lingonberry belongs to the family Ericaceae (common name: Heath Family). The Ericaceae includes more than 100 genera with slightly more than 3000 species. This family has a worldwide distribution and is mostly composed of shrubs with some climbing plant species (Heywood 1978). Certain members of this family, including lingonberry have evergreen leaves that are adapted to cold, dry environments and have a thick leathery cuticle and a reduced surface area (Penhallegon 2006).

The lingonberry belongs to the Ericaceae sub-family Vaccinioideae, and is placed in the Genus *Vaccinium*. *Vaccinium* is derived from the Latin roots "vacci" and "nium", translating roughly to "pertaining to cows" (Greuter *et al.* 2000). North America is home to 26 native species of *Vaccinium*, nine of which are native to Newfoundland and Labrador including the commercially important *V. angustifolium* (lowbush blueberry), *V. macrocarpon* (cranberry) and *V. myrtilloides* (common or velvet leaf blueberry). The genus *Vaccinium* is native to all continents with the exception of Antarctica and Australia. There are several isolated islands to which plants of this genus of plant are non-native (Vander Kloet 1988).

Two subspecies of lingonberry are recognized: *V. vitis-idaea* ssp. *vitis-idaea* (L.) Briton and *V. vitis-idaea* ssp. *minus* (Lodd.) Hult. (Hulten 1949, Penhallegon 2006) distinguished primarily by their size at maturity (Figure 1.1). The maximum plant height observed in ssp. *minus* (native to Newfoundland) is on average less than 20 cm whereas ssp. *vitis-idaea* L. (European) can reach heights of more than 30 cm or more. Leaf size is much smaller is subspecies *minus* than in the European subspecies (Fernald 1970). Leaf size at maturity can be used as an identifying characteristic. Leaves of *V. vitis-idaea* L. ssp. *vitis-idaea* averages leaves 2.5 cm in length and 1.0 cm in width, *V. vitis-idaea* ssp. *minus* leaves are on average 1.0 cm in length and 0.5 cm in width (Welsh 1974). The research in this paper focuses on *V. vitis-idaea* ssp. *vitis-idaea* unless otherwise noted.

Figure 1.2: Open pollinated greenhouse-grown lingonberry fruit

1.3 Distribution

In accordance with its wide geographical distribution, *V. vitis-idaea* has good adaptability and can survive in a range of environments. Common environments for lingonberry are sandy, northern, temperate boreal forests (Penhallegon 2006). Soils where wild lingonberries are common are often infertile and rocky, but the plants can also be found in loams (Small *et al.* 2003), bogs, moors, headlands and both cliff and mountain summits (Vander Kloet 1988). The plants can suffer 'die back' in the absence of adequate snow cover in the winter, early bud development, exposed flowers and fruit are susceptible to frost damage in sub-zero temperatures (Vander Kloet and Hall 1981). Lingonberries grow well in light soil with good drainage and an acidic pH in the range of 4.3 to 5.5, due to their cool climate adaptability. Lingonberry plants grow often with little competition from other vegetation due to the harsh environments to which they are native. They can be found typically in regions where blueberries are common, although they grow in separate and distinct micro-habitats (Ailor and Penhallegon 1999).

Lingonberry has a circumpolar and circumboreal distribution (Trajkovski 1987, Small *et al.* 2003). Subspecies *vitis-idaea* is distributed primarily in northern temperate, boreal and subarctic regions (Gustavsson 2001). Twenty four countries currently have natural stands of wild lingonberries (ssp. *vitis-idaea*) of many varieties, mostly in Europe. Many new countries are starting cultivation programmes to introduce the lingonberry as a sustainable crop (Penhallegon 2006). Newfoundland and Labrador is the largest North American producer of *V. vitis-idaea* ssp. *minus* with an annual harvest of 140,000 kg, all

from native stands (Jamieson 2001). The Newfoundland *V. vitis-idaea* var. *minus* harvest peaked in 1994 when harvested fruit exceeded 400,000 kg while in 2003 the harvest was only 50,000 kg (Ricketts 2004). The annual variation and unpredictability of the native lingonberry harvest in Newfoundland and Labrador has been one of the most significant obstacles to establishing cultivation programmes and industry in the province. The world's largest lingonberry producer is Sweden, with harvests of ssp. *vitis-idaea* exceeding 20,000 tonnes in some years from both wild and cultivated sources (Penhallegon 2006).

1.4 Biochemical Properties

Lingonberries contain vitamin C, provitamin A (as beta carotene), B vitamins (B_1, B_2, B_3), as well as various macro and micronutrients (Oldemeyer and Seemel 1976). Lingonberry has been reported to have high levels of antioxidants primarily in the form of anthocyanins (Figure 1.3) (Wang *et al.* 2005). Certain anthocyanins are considered anti-cancer flavonoids when consumed in the human diet (Stark *et al.* 1978), they are responsible for the dark colouration of the lingonberry.

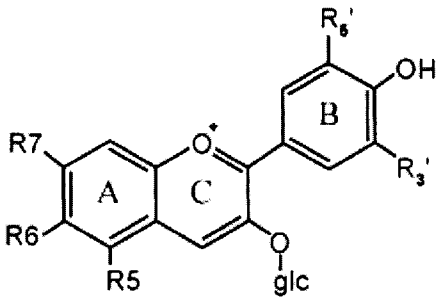

Figure 1.3 : General chemical structure of anthocyanins. Position 3 marks the position to which the sugar moiety binds to the carbon skeleton (glc represents glucose)

The protein content of lingonberry fruit remains constant at approximately 5.5% so long as the fruit is attached to the branch, is ripe and undamaged. While iron, magnesium and zinc content in lingonberry fruit declines sharply over winter, the content of other macronutrients, as well as fibre and lignin increase as winter approaches (Oldemeyer and Seemel 1976). The energy content of the lingonberry browse has been estimated to be 509 Kcal per 100 g (Miller 1976). The nutritional value of Alaskan lingonberries was evaluated; the results show distinct seasonal variation (Oldemeyer and Seemel 1976).

1.4.1 Anthocyanin

Phenolics are a large and diverse chemical group (Duy 1999). Flavonoids are a subgroup of the phenolics, to which anthocyanins belong. Anthocyanins have a 15-carbon skeleton, with a carbohydrate attached at position 3 (Figure 1.3). One of the most important fruit characters of lingonberry fruit is its colour. The bright red colour observed is due to the presence of anthocyanins, a class of water-soluble flavonoids commonly found in plants. There are over 300 different anthocyanins identified in terrestrial plants, they are the most common class of pigmented compounds in plants (Jones *et al.* 2003). Of the six common anthocyanins found in plants (Figure 1.4), only cyanidin occurs in lingonberries (Kahkonen *et al.* 2003).

Lingonberries have a distinctive pattern of anthocyanin distribution, reported by Kahkonen *et al.* (2003) and Wang *et al.* (2005). Cyanidin-3-galactoside is the predominant anthocyanin in lingonberry and accounts for over 80% of the total anthocyanins and contributes the most antioxidant activity (Wang *et al.* 2005) while the remaining 17% is divided between cyanidin-3-glucoside (5%) and cyanidin-3-arabinoside (11%).

Anthocyanins serve two primary functions in plants. First; as a result of their bright colouration the flowers are involved in insect attraction for pollination. The pink and white colours of lingonberry flowers, which are due to anthocyanin, make them easily visible to bumble bees and other insect pollinators of lingonberries (Davis *et al.* 2003). Their secondary function is to serve as an ultraviolet (UV) screen to protect the

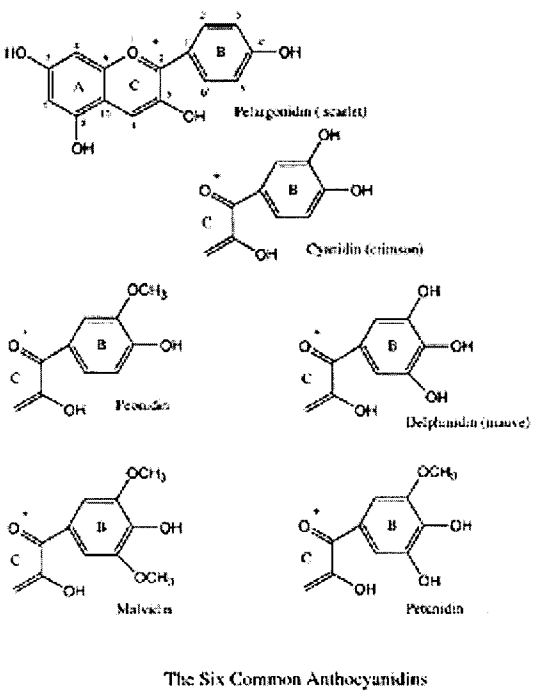

Figure 1.4: The six most common anthocyanins in terrestrial land plants: pelagronidin, cyanidin, peonidin, delphinidin, malvidin and petunidin

plant from UV radiation. They protect the plant's DNA from damage by sunlight (McClure 1975). UV light may cause mutations via oxidative damage to the genetic material within the plant preventing essential processes such as protein synthesis, hormone-related signalling and cell division (Duy 1999). Anthocyanins are not found in aquatic plants; this has lead to the theory that anthocyanins are an evolutionary adaptation to living on the land where UV radiation is a constant threat (Schaefer and Wilkinson 2004). Anthocyanin biosynthesis is a complex yet well documented process; it requires multiple reactions with many intermediate steps. The production of anthocyanins requires two simultaneous pathways to work together and eventually merge to produce the anthocyanin. The metabolism of photosynthetically-derived CO_2 produces a C_2 molecule and is the starting material for both pathways. One pathway ultimately produces the amino acid phenylalanine, while the other produces a three carbon molecule of malonyl-Coenzyme A. Enzymes join the two compounds together to produce a compound called naringenin, which, with the help of other enzymes produce a final anthocyanin. The production of phenolic compounds, including anthocyanins appears to be a response to environmental stimulus (Bohm 1987). There is evidence to suggest that anthocyanin production is a stress response to cold and other factors (Chalker-Scott *et al.* 1989).

1.4.2 Antioxidants

Antioxidants are defined as any substance that reduces oxidative damage such as that caused by free radicals (Duy 1999). Free radicals are highly reactive compounds that modify molecules by capturing electrons and thus modify chemical structures in modifying ways. Plants have many sources of antioxidants: anthocyanins, enzymes, vitamins, and other phenolic compounds have effective free radical scavenging properties (Ziccarelli 2001).

There are two major modes of antioxidant activity, the first and simplest being hydrogen donation, where an antioxidant molecule donates an electron to a free radical preventing it from scavenging the electron from important cellular machinery or DNA. The second mechanism of action is the formation of stable complexes with free radicals, and in doing so eliminating the damage associated with free radical activity. The role of antioxidants is to nullify the threat of oxidative damage and electron scavenging associated with free radical formation.

There are endogenous antioxidants in the body that serve to protect against oxidative damage from free radials and there are exogenous sources of antioxidants obtained exclusively from the diet (Betteridge 2000). Endogenous sources of antioxidants are categorized as enzymatic and non-enzymatic (Zicarelli 2001). Exogenous antioxidants are defined as antioxidants provided exclusively from the diet that act to compliment the body's endogenous defence system (Betteridge 2000). Lingonberries provide exogenous antioxidants in the form of cyanidin glycosides, tocopherol, vitamins

and carotenoids, all of which contribute to the overall antioxidant capacity of lingonberries (Wang *et al.* 1997).

1.5 Health Benefits of Lingonberry

Biochemical analysis and pharmaceutical research has yielded a great deal of information on the use of lingonberry as a medicinal plant. Chiej (1984) reported that lingonberry leaves could be used as an antiseptic and as a astringent. The leaves also have diuretic properties (Lust 1983) and can be used to relieve symptoms from the common cold such as a sore throat (Moerman 1998). Lingonberry has also been used in the treatment of the sexually transmitted infection gonorrhoea (Duke ad Ayensu 1985). The leaf extracts are commonly used in the treatment of rheumatic disease such as arthritis and rheumatism, as well as diabetes and diarrhea (Launert 1981). Lingonberry leaves contain high levels of arbutin which is thought act as a anti-microbial agent (Frohne 1970), help treat and prevent urinary tract infections (UTI's) (Larsson *et al.* 1993), treat stomach disorers (Racz *et al.* 1962) and assist in melanogenesis inhibition which in turn reduces the occurrence of skin conditions such as chloasma when applied topically (Sugai 1992, Matasuda *et al.* 1996). However, it should be noted that when consumed in elevated levels, arbutin is toxic and it is thought to be a causal factor in leukemia development (McDonald *et al.* 2001). There are many ways to utilize lingonberries medicinally; when consumed raw the fruit stimulates the production of gastric juices that aid in digestion and immune system functionality. Fruits and leaves can also be used

medicinally as kidney and bladder disinfectants and to lower cholesterol (Dierking and Dierking 1993). Koide *et al.* 1996 reports the antitumor effects of anthocyanins, including cyanidin, the primary anthocyanin found in lingonberries.

1.6 Uses of Lingonberry

Lingonberry has a wide range of uses: jams, juices, sauces, preserves, candies, wines, liqueurs and ice creams (Gustavsson 1997). More recently, lingonberry extracts have been used in holistic medicines, dietary supplements and topical creams promoting skin care (Liebster 1975, Holloway 1985). Although there are reports about detrimental nature of arbutin to humans, it is also thought to be involved in melanin formation and implicated in melanoma prevention (Matasuda *et al.* 1996). Aside from neutraceutical and pharmaceutical uses, lingonberry is used commonly as ornamental ground cover in landscaping mostly in Europe (Dierking and Dierking 1993). Lingonberry is an important foraging crop for many animals such as black bears, foxes, moose, squirrels, chipmunks, hares, skunks, Canada goose and other birds even polar bears. Because the berries remain on the branches through the winter they are especially useful to animals that can dig through snow to get to them.

1.7 Lingonberry Propagation

Plant micropropagation is the production of new plants from small pieces of plant (cells, tissues, organs) in a relatively short period of time using tissue culture techniques. Plant propagation, in the general sense, is the multiplication of plant material. As demand increases for lingonberry fruit from industry and global consumers, the importance of commercial propagation increases as well. The lingonberry is commonly propagated by conventional means, either from seed or vegetatively by stem cuttings and rhizome division but can also be propagated by micropropagation (Penhallegon 2006).

1.7.1 Propagation from Seed

Sexual propagation in plants involves the exchange of genetic material between two plants resulting in a seed that will produce a new plant. Propagating plants by seed is the most commonly used method of plant production in self pollinated crops and where vegetative propagation is not possible.

1.7.2 Vegetative (*ex vitro*) Propagation of Lingonberry

Vegetative methods of propagation involve cuttings from stem, root and rhizome material. Cuttings are of two main types, softwood or hardwood depending on the developmental stage and age of the selected material. Cuttings generally involve removing a piece of a parental plant, and having that piece re-grow into an independent adult plant.

1.8 Conventional Vegetative Propagation Techniques

Conventional techniques have long been a common and successful agricultural practice; parental plant material is multiplied and propagated to produce 'clones', often in large numbers. Softwood cuttings refer to cuttings taken from young, first year branches that have not yet matured (Figure 1.5). The plant material selected for propagation must have a meristem to produces axillary shoots and a section of stem from which adventitious roots will develop. A meristem contains all the vegetative cells from which a plant will develop. This meristem is divided into distinct regions each having a specific role in the assignment of cell fate and the differentiation of the cells in the growing region into the appropriate structures (Wolpert *et al.* 2002). The cutting can be treated with growth hormones (generally an auxin such as Indole-3-butyric acid 'IBA') or grown without hormonal supplementation in the soil (Debnath 2006). The softwood cutting method requires three to five centimetre cuttings on average, and several weeks for the

plant to acclimate to the soil. The technique is time consuming for multiplying large numbers of plants from a single genotype, a large amount of starting material is required. Once planted, both TC and SC plants require a regular irrigation schedule, this is important to ensure the young plants receive enough water. Furthermore, healthy soil and fertilization treatments take care of nutrient requirements, while weeding and pot maintenance (such as moss removal and insect pest management (IPM)) make sure that the plant will not be out-competed by weeds or suffocated by detritus. The alternative to softwood cuttings is hardwood cuttings, which refers to cuttings taken once the plant tissue becomes woody; typically at the stage plants are dormant.

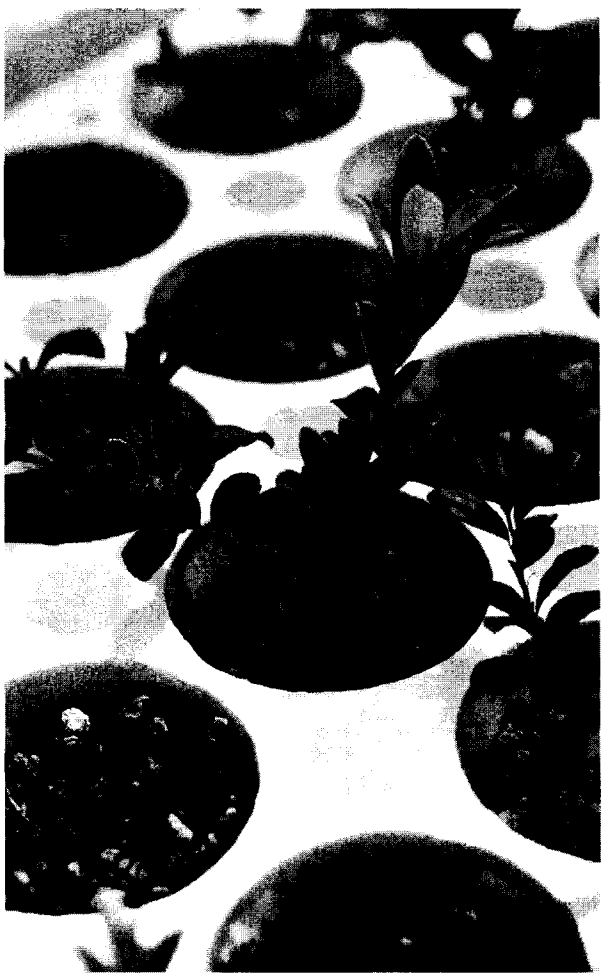

Figure 1.5: Conventional softwood stem cutting-propagated lingonberry cultivar 'Erntedank'

roots. At this point the plant can resume development and begin growing anew. The same process applies to both leaves and roots.

Rhizomatous divisions are another form of vegetative production that is commonly used to propagate *Vaccinium* species.

1.9 Tissue Culture Propagation of Lingonberry

Plant tissue culture refers to the aseptic culture of any plant part under *in vitro* conditions. *In vitro* is Latin and means "in glass"; it refers to the artificial environment created outside a living organism generally in a test tube or glass container. This contrasts the term *ex vitro*, which means "from glass". Plant micropropagation is the production of new plants from small pieces of plant (cells, tissues, organs) in a relatively short period of time using tissue culture techniques. Tissue culture systems are a widely-used and efficient micropropagation system for many plant species important to agriculture, research and industry (Hosier 1985). Generally the medium for *in vitro* culture consists of water, micronutrients and macronutrients, some carbon source (usually carbohydrates in the form of sucrose or glucose), vitamins, growth regulators (auxins, cytokinins and gibberellins) and a chelating agent (in the case of solid medium). All these components act together in synergy under aseptic conditions to form a microenvironment that allows for plant growth (Debnath and McRae 2001). The benefits of tissue culture are numerous: the production of disease and pathogen-free plantlets, the rapid production of large numbers of genetically identical plantlets, year round plantlet production, germplasm preservation and the creation of variability (Debnath 2003).

Two concepts that are fundamentally important to the understanding of plant cell culture and micropropagation are that of plasticity and totipotency. Plasticity is the ability of plant material to adapt to different environmental conditions by altering metabolic functions, development and chemical composition. The high level of plasticity exhibited

by lingonberry is exploited in tissue cultured systems to initiate the regeneration of stem or root tissue from segmented vegetative or somatic tissue. Totipotency is the potential of all plant cells to perform all the functions of development required to regenerate a new plant. This ability allows for a single cultured plant cell to produce an entire plant *in vitro*, a clone (Wolpert *et al.* 2002). *In vitro* technology can be used to compliment conventional techniques of plant breeding. Tissue culture can rapidly and aseptically produce large amounts of plant material, while selecting for and cloning superior germplasms that are disease-resistant and produce elevated levels of vegetative growth. Complete new plants can be derived from tissue culture in three ways: through axillary shoot proliferations, adventitious shoot regenerations and from somatic embryogenesis.

1.10 Methods of *in vitro* Propagation

1.10.1 Axillary Shoot Proliferation

Every plant cell has the potential to become the starting material required to grow into a new plant, a clone of the parental material. The choice of starting material in tissue culture determines the path the explant will go through to produce new shoots and plants. Young, actively-growing shoots are best suited for tissue culture. Typically, nodal explants initially contain one or more node segments. The segments are treated aseptically as described in Debnath and McRae (2001). Axillary shoot development from nodal explants is similar to that in conventional stem cuttings in that they arise from the

buds located at the nodes. Generally these buds are dormant or inactive as a result of hormonal and genetic interactions, these signals arise from the active apical meristem and act to inhibit lateral bud activation (Wolpert *et al.* 2002). In the absence of an inhibitory signal from the apical meristem, such is the case when nodal explants are segmented, lateral bud activation occurs and genetic mechanisms responsible for shoot development are activated.

1.10.2 Adventitious Shoot Regeneration

The ability to generate shoots from somatic plant tissue in a reliable fashion would allow for manipulation of the lingonberry genetic make-up and produce new cultivars having beneficial or desirable characters (Debnath and McRae 2002). Leaf explants in tissue culture systems rely on the totipotency of cells to produce new adventitious shoots resulting in new plantlets. Adventitious shoot regeneration occurs only following plant tissue dedifferentiation and reorganization into meristimatic tissues with or without an intermediate callus stage (Litz and Gray 1992, Debnath and McRae 2002). It is at the intermediate callus stage of development that somaclonal variation is most likely to occur (Larkin and Snowcroft 1981). Studies have shown that the most distal segments of leaves produce the greatest regeneration capacity as a result of having more active meristematic cells in that region (Yepes and Aldwinckle 1994). The production of shoots from plant tissue whose fate had been set as non-stem tissue introduces the possibility of genetic transformations either beneficial or detrimental to the

fitness of the plant. Previous research has shown that somaclonal variation "frequently" produces undesirable variants (George 1996). Generating shoots from somatic plant tissues using tissue culture would allow for identification and mutiplication of somaclonal variants (Rosati *et al.* 1990).

Until 2002 there was no documented regeneration of adventitious shoots from lingonberry. Debnath and McRae (2002) first reported an efficient system for the regeneration of adventitious shoots from excised leaves of lingonberry. Previous to that study, excised leaves of the *Vaccinium* species' highbush blueberry, half-high blueberry and cranberry had been used to regenerate new plants from leaf segments. Subsequently, the technique has been applied to many species of commercial importance including apples, roses, and strawberry (Debnath 2005a). There is general agreement that *in vitro* shoot regeneration is affected by genotype, basal medium and physical conditions (i.e., light and temperature) (Yildiz and Er 2002).

1.10.3 Somatic Embryogenesis

Thorpe (1988) defines somatic embryogenesis as the development of haploid or diploid cells into differentiated plants through embryo stages without the fusion of gametes. Somatic embryogenesis is an asexual form of plant reproduction that can be manipulated under *in vitro* conditions. Somatic embryos are formed from protoplasts or small amounts of plant material not normally associated with embryogenesis; generally at the callus stage during *in vitro* culture.

1.11 Morphological Characters of Tissue Culture Derived Plants

In vitro systems produce plants with dense vegetative growth patterns, increased rhizomatous shoot proliferation, and increased branching (Debnath 2005b). Holloway (1985) and Debnath (2005b) reported that lingonberry plants propagated by softwood cuttings generally fail to produce rhizomes and rhizomatous shoots even after prolonged periods of time; whereas plants propagated *in vitro* showed high levels of rhizome and rhizomatous shoot production often in less than one year of growth (Serres *et al.* 1993). In previous studies on lingonberry, tissue culture-derived plants were more vigorously branched and heavily rooted than plants produced by conventional softwood cuttings (Holloway 1985). Plants produced via adventitious shoot regenerations have shown denser canopy production, increased vegetative shoot development and significantly elevated production of dense rhizome networks when compared to conventional softwood cuttings (Debnath 2006).

1.12 Applications of Micropropagation

The uses of micropropagation techniques are broad and variable, from the fields of agriculture, biology and biotechnology to medicinal applications. In agriculture, the rapid germplasm multiplication made possible by tissue culture techniques reduces the time required to prepare crops for planting and provides high yielding, healthier crops to the farming industry. This technology also secures the ability to meet the rising global

food demand by producing sustainable food crops in great numbers. The biotechnology industry is interested in tissue culture practices due to the rapid production of a large number of genetically identical crops. The neutraceutical and pharmaceutical industry use many plant species in research for medicinal extracts and uses. Therefore it is of paramount importance that plants studied are pathogen free, identical and reproducible.

1.13 Lingonberry Industry in North America

From reports published in 2005 by the Oregon Agricultural Statistics Service the average yield per acre of the lingonberry from 2004 was just below 3,000 pounds with an average value of utilized production of over $2,100.00 per acre in Oregon (Oregon Agricultural Statistics Service Crop Report 2005). Existing markets are putting increased pressure on producers and exporters for higher quality fruit, both cosmetically (fruit are large, firm and clean) and from a health point of view (fruit are high in antioxidants, vitamins and fibre). Commercialization of cultivated lingonberry fields that would be able to provide a steady supply of such fruit in Newfoundland and Labrador is feasible and potentially profitable. Currently 17 acres, or one quarter of the world's cultivated lingonberry acreage is in Oregon, Washington and British Columbia. Smaller regions of lingonberry farming exist elsewhere in North America; however a majority of lingonberry cultivation remains in Europe (Penhallegon 2006). Only slightly more than 10% of wild lingonberry is harvested on average, no more than 40% in some regions. The lack of efficient harvest procedures and high level of un-harvested fruit have resulted in

reduced interest in developing agricultural practices for this fruit (Burt and Penhallegon 2003). However increasing demand and high return rates on harvested fruit make cultivation of this crop economically viable and profitable.

1.14 Research Objectives

The objective of the present study was to evaluate and compare the performance of two lingonberry cultivars propagated by conventional stem cuttings with those obtained by adventitious shoot regeneration from excised leaves.

This research will help understand the extent to which propagation method effects lingonberry plants in terms of morphology as well as chemical composition. The synthesis of chlorophyll in the leaves and anthocyanin content as well as the antioxidant activity in the fruit were evaluated within and between cultivars having been propagated using conventional stem cuttings and adventitious shoot regenerations. The goal of this research was to develop a more productive, efficient and sustainable propagation system for lingonberry plants in Newfoundland and Labrador.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant Material

The study was conducted with greenhouse-grown lingonberry cultivars 'Splendor' and 'Erntedank' at the Atlantic Cool Climate Crop Research Center (ACCCRC) in St. John's, Newfoundland and Labrador, Canada.

'Splendor' is a high yielding American cultivar which after several years of growth produces fruit known to have high anthocyanin content; 'Splendor' shows moderate levels of frost resistance and acceptable has vigour (Stang 1994). 'Splendor' was first developed in 1987 from open pollinated seed in Finland. Originally named WI102 this cultivar was grown in Wisconsin in the spring of 1988, it was selected from a pool of seedlings numbering over 3000 based on plant vigor, apparent cold tolerance and the absence of leaf disease (Stang 1994). 'Splendor' has two blooms, a far greater yield is obtained from the second and fruit of the first are generally discarded due to low yield.

The 'Erntedank' cultivar is German in origin and is high yielding with good spreading abilities through its rhizomes (Zillmer 1985). 'Erntedank' was developed in 1985, this cultivar produces small fruit, it grows moderately well and has good cold tolerance (Penhallegon 2006).

2.2 *In vitro* Culture Establishment and Adventitious Shoot Regeneration

In June 2000, following the protocol of Debnath and McRae (2001), cultures of lingonberry cultivars 'Splendor' and 'Erntedank' were established *in vitro* in 175 ml Sigma baby food glass jars containing 35 ml BM medium [(three-quarter macro-salts and micro-salts of Debnath and McRae's (2001) shoot proliferation medium D)] supplemented with 25 g L^{-1} sucrose, 5 μM zeatin, 3.5 g L^{-1} Sigma A 1296 agar (Sigma Chemical Co., St. Louis, MO) and 1.25 g L^{-1} Gelrite (Sigma Chemical Co., St. Louis, MO). The medium pH was adjusted to 5.0 before autoclaving at 121°C for 20 min. Cultures were maintained at 20°C ± 2 under a 16 hour photoperiod [(photosynthetic photon flux (PPF) density of 30 µmol m^{-2} s^{-1} at the culture level)] provided by cool white fluorescent lamps and sub-cultured every 8 weeks until January 2001 when the shoot regeneration experiment started.

Adventitious shoot regeneration was obtained from leaf explants of proliferating shoot cultures (Debnath and McRae 2002, Debnath 2005c). Buds and shoot clumps regenerated from leaf explants on BM supplemented with 5 µM zeatin were collected eight weeks following culture initiation and transferred to 175 ml Sigma baby food glass vessels containing 35 ml of BM with zeatin (1 µM) and cultured for another 8 weeks for shoot elongation at the same photoperiod, light intensity and temperature employed previously for shoot proliferation.

2.3 Rooting, Acclimatization and Evaluation for Morphological Characters

Rooted softwood stem cuttings (SC) and adventitious shoots obtained from tissue culture (TC) of 'Splendor' and 'Erntedank' were grown in the greenhouse following Debnath (2006). Eight week old *in vitro* derived elongated shoots (4 to 5 cm long) were rooted in 45-cell plug trays (cell diameter: 5.9 cm, cell depth: 15.1 cm; Beaver Plastics, Edmonton, AB) containing 2 peat: 1 perlite (v/v) in a humidity chamber with a vaporizer (Conviron E15, Controlled Environments Ltd., Winnipeg, MB) at 22 ± 2°C, 95% RH at 16 hour photoperiod at 55 μmol m^{-2} s^{-1} PPF. No rooting compound was applied.

Greenhouse-grown plants used for TC provided SC stems for rooting. At the same time as *in vitro* culture establishment in May, 2001, terminal softwood cuttings 4 to 5 cm long were taken from new growth of greenhouse-grown 'Splendor' and 'Erntedank' plants used for TC and prepared as previously described for rooting of TC microcuttings in 45-cell plug trays containing the same potting medium. After 8 weeks the rooted SC and TC plantlets were transferred to 10.5 (L) × 10.5 (W) × 12.5 (D) cm^3 plastic pots containing the same medium as used for rooting, maintained in the humidity chamber and acclimatized by gradually lowering the humidity (3-4% weekly) over 3 weeks. Hardened-off plants were grown in the greenhouse under natural light conditions at a maximum PPF of 90 μmol m^{-2} s^{-1} at 20 ± 2°C, 85% RH. Fertilizer 20-8-20 N-P-K (Plant Products Co. Ltd., Brampton, ON) and irrigation was applied when necessary (Debnath 2006). Maintaining the plants at or below 5°C for 12 weeks induced dormancy. After a second and third 12-week dormancy period from January to March, 2003 and 2004, respectively,

the plants were grown in the greenhouse until data on stem number, primary, secondary and total branch number, plant vigour, leaf number per plant, leaf number per stem and plant height were taken in December, 2004. Vigour was defined as a measure of each plants overall health and was rated categorically on a scale of 1 – 8, with 1 being discoloured and dying and 8 being brightly coloured and healthy. The average day length was 13.4, 15.0, 15.5, 15.3, 14.1, 12.3, 10.5, 9.2 and 8.3 hours, for months April to December, respectively (http://aa.usno.navy.mil/cgi-bin/aa_rstablew.pl). The experiment was arranged in randomized block design, with five plants per treatment, and the experiment was repeated three times with a total of 60 plants.

2.4 Leaf Chlorophyll Estimation

Chlorophyll a, b and chlorophyll a + b were analyzed according to the procedures of Porra (2002). All leaf disks were collected in June 2005 after four years of plant growth. Three replications of five samples were collected for a total of fifteen plants per treatment. A standard one-hole punch was used to remove a leaf disk from the center of leaves still attached by the petiole to the branch. All leaves sampled were taken from the tip of the branch. Taking leaves of similar developmental stage and health ensured obtaining sample homogeneity, the same hole punch was used to ensure consistent sample size. The leaf discs (0.01 g) were immediately placed in sterile 2 ml centrifuge tubes, capped and placed on ice until used for analysis. The leaf disks were manually

homogenized in 5 ml of 80% acetone using a mortar and pestle, centrifuged at 5000 rpm for 1 minute and 1 ml of supernatant was analysed using a UV/VIS spectrophotometer (Pharmacia Biotech Ultrospec 2000) at 645 nm and 663 nm for chlorophylls a and b respectively. The chlorophyll was quantified using the following formulae:

$$\text{Chlorophyll } \mathbf{a} \ (\mu g/ml) = 12.25\ (A_{663.6}) - 2.55\ (A_{646.6})$$

$$\text{Chlorophyll } \mathbf{b} \ (\mu g/ml) = 20.31\ (A_{646.6}) - 4.91\ (A_{663.6})$$

$$\text{Total chlorophyll } (\mu g/ml) = 17.76\ (A_{646.6}) + 7.34\ (A_{663.6})$$

2.5 Fruit Extracts for Anthocyanin Content and Antioxidant Activity Analysis

Mature fruit with well-developed red colour were manually harvested in August and again in October 2004 and immediately frozen at -20 °C until analysis. Ripeness was defined as the point where the entire fruit was dark red in colour with no white or pink anywhere on the fruit. Four grams of the berries from each replication of all treatments were homogenized in 5 ml of ethanol: 1.5 N HCl (85 : 15, v/v) to extract anthocyanins and left overnight at 4 °C. The extracts were filtered through 0.2 μM syringe filters before analysis to remove cellular debris.

2.6 Total Fruit Anthocyanin

The total anthocyanin content of the fruit extract was measured using the pH differential method (Jones *et al.* 2003). This method estimates total anthocyanin content based on the reversible conversion of anthocyanins from the oxonium to the hemiketal form, but does not determine the individual compounds present. Absorbance was measured in the Pharmacia Biotech Ultrospec 2000 spectrophotometer at 510 and 700 nm in triplicate in buffers at pH 1.0 and pH 4.5. The difference between these two values was used to determine the total anthocyanin concentration based on a cyanidin-3-glucoside molar extinction coefficient of 26900 and a molecular weight of 449.2 (Jones *et al.* 2003). Values were expressed in terms of mg of anthocyanin per 100 g of frozen fruit. The values were then quantified using the following formula:

$$\text{Total anthocyanins} = A \times MW \times DF \times 10^3 \times \varepsilon^{-1} \times 1^{-1}$$

where: $A = (A_{520nm} - A7_{00nm})_{pH\ 1.0} - (A_{520nm}\text{-}A_{700nm})_{pH4.5}$; MW (molecular weight) = 449.2 g per mol; DF (Dilution Factor); 1 = pathlength in cm; ε = 26900 molar extinction coefficient ($L \times mol^{-1} \times cm^{-1}$) and 10^3 = conversion factor from g to mg.

2.7 High-Performance Liquid Chromatography (HPLC) Separation of Anthocyanins

Anthocyanins were separated by HPLC following Zhou and Singh (2004) on an Agilent 1100 series LC/MSD trap system (Agilent Technologies, Palo Alto, CA)

controlled by ChemStation software (Agilent, version 08.03) and equipped with a 1024-element diode-array detector and a C_{18} column (4.6 × 150 mm) with 5 μM particle size (Chromatographic Specialties, Brockville, On.). Elution was carried out using a mobile phase formed by a linear gradient of H_20 - acetic acid (10 : 1) (A) and methanol - acetic acid (10 : 1) (B), with 100% (A) at 0 min to 40% (A) and 60% (B) at 20 min. The flow rate was fixed at 0.2 mL min^{-1} and the detection wavelength was 535 nm. Anthocyanin content was measured in absolute quantities using the extinction coefficient (ε^1_{1cm}) at 535 nm as 98.2 (Francis 1982).

2.8 Antioxidant Activity

The antioxidant activity was measured using the anti-radical power (ARP) method following Hanson *et al.* (2004). The method is based on the capacity of different components to scavenge the ABTS (2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)) radical cation compared to a standard antioxidant Trolox, a vitamin E analogue, in a dose–response curve. The antioxidant activity of a sample for the ARP assay was measured within the linear relationship of concentration versus optical density decrease, and presented as Trolox equivalent (TE) in μmol g^{-1} fruit (frozen weight basis).

2.9 Statistical Analysis

Data for all characteristics except shoot vigour were subjected to analysis of variance with the Statistical Analysis Software (SAS) package (Release 8.2, SAS Institute, Inc., Cary, NC). Morphological characters, except plant height were square root transformed, prior to the ANOVA to stabilize the variance and then back-transformed for presentation. Differences among treatments were further analyzed using Duncan's multiple range test. Categorical data modelling (CATMOD) analysis was performed on qualitative data such as plant vigour; any difference between treatments was assessed using the contrast statement in the CATMOD procedure. This method yields similar results to ANOVA and is suitable for qualitative data (Compton 1994).

CHAPTER 3: RESULTS

3.1 Morphological Characters

After four years of growth under greenhouse conditions both SC and TC plants showed significant vegetative growth but had very different morphologies (Table 1). Analysis of variance (ANOVA) indicated significant interaction between cultivar and propagation method for secondary branch number, total branch number and leaf number per plant (Table 1). It should be noted that the plants in these experiments belong to AAFC and were grown for 2 years prior to the beginning of this thesis. The morphological development of lingonberry plants varied significantly between cultivars for stem number, primary branch number, secondary branch number, total branch number, leaf number per plant, leaf number per stem and plant height. Across cultivars 'Erntedank' produced more stems, branches (primary and secondary), and produced more leaves per plant and per stem than those of 'Splendor' although 'Splendor' plants did achieve greater average plant height (Table 1).

Propagation methods had significant affects on all morphological characters except secondary branch number and plant vigour (Table 1). TC plants developed more stems, primary branches and leaves than SC plants. However, SC plants were taller, with more secondary and total branches (Table 1). Figure 3.1 shows the cultivar-specific response to propagation method for stem number. SC plants produce rarely more than one primary stem and few if any rhizomatous shoots whereas TC plants have

significantly greater numbers of rhizomatously generated primary stems. Figure 3.2 shows that for primary branch number, TC plants were significantly superior to SC plants. Secondary branch number was also significantly different between cultivars (Figure 3.3), as well as total branch number (Figure 3.4). The highest plant heights obtained in this population were found in the TC group for both cultivars (Figure 3.5). Both TC and SC 'Erntedank' plants produced a superior number of leaves per plant than those of 'Splendor' (Table 1, Figure 3.6).

Table 1: Effects of cultivar and propagation method (SC = stem cutting, TC = tissue culture) on the morphological characters of two lingonberry cultivars measured after four growing seasons.

	Stem number per plant	Primary branch number per plant	Secondary branch number per plant	Total branch number per plant	Plant vigour (1-8)	Leaf number per plant	Leaf number per stem	Plant height (mm)
Analysis of variance				P values				
Cultivar (CV)	0.0373	0.0456	<0.0001	0.0027	0.8351	0.0190	0.0002	<0.0001
Propagation method (PM)	<0.0001	<0.0001	0.0854	0.0053	0.4808	<0.0001	0.0009	0.0001
CV × PM	0.0861	0.9733	<0.0001	0.0004	0.4808	0.0047	0.2783	0.0927
Means								
Cultivar								
'Splendor'	1.04b	2.13b	5.67b	7.73b	4.63a	140.19b	18.15b	105.47a
'Erntedank'	5.38a	4.08a	7.72a	12.40a	4.77a	297.56a	24.60a	78.20b
Propagation method								
SC	1.00b	2.78b	5.20a	8.18a	4.9a	213.16b	26.11b	132.40a
TC	4.21a	4.92a	1.61b	7.62b	4.5a	288.32a	41.34a	121.13b

Means within columns and factors with different letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test.

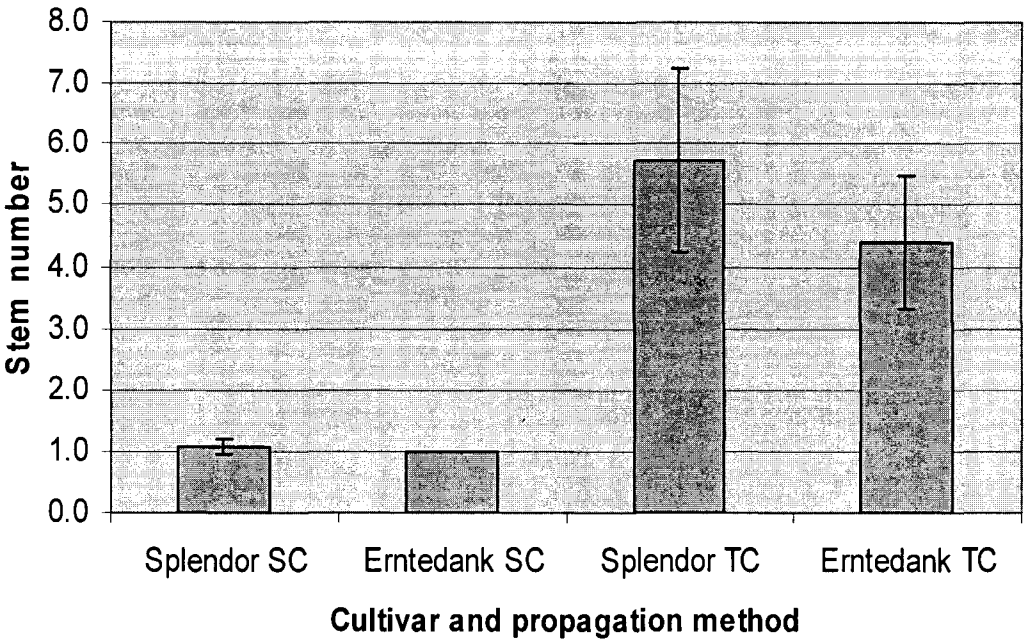

Figure 3.1: Number of stems in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

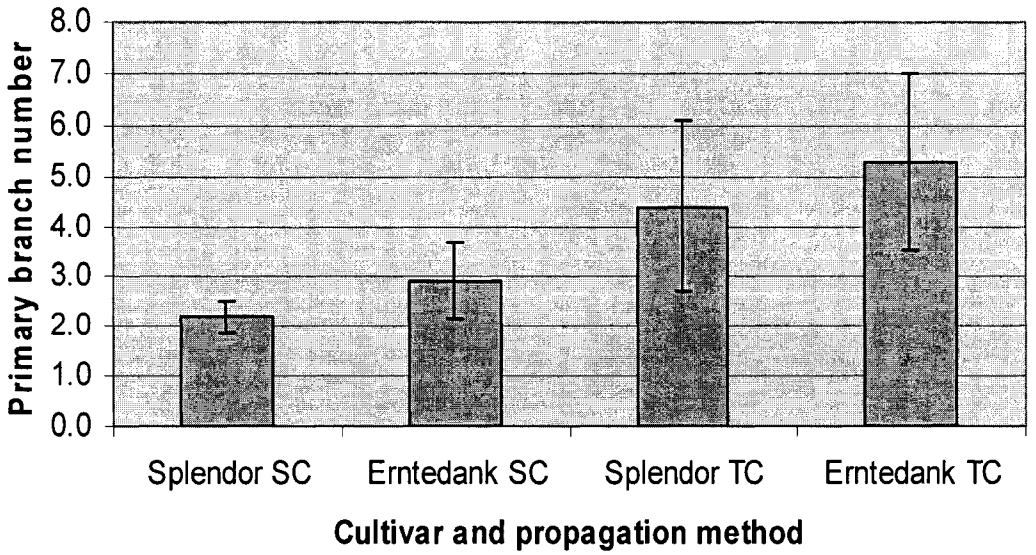

Figure 3.2: Number of primary branches in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

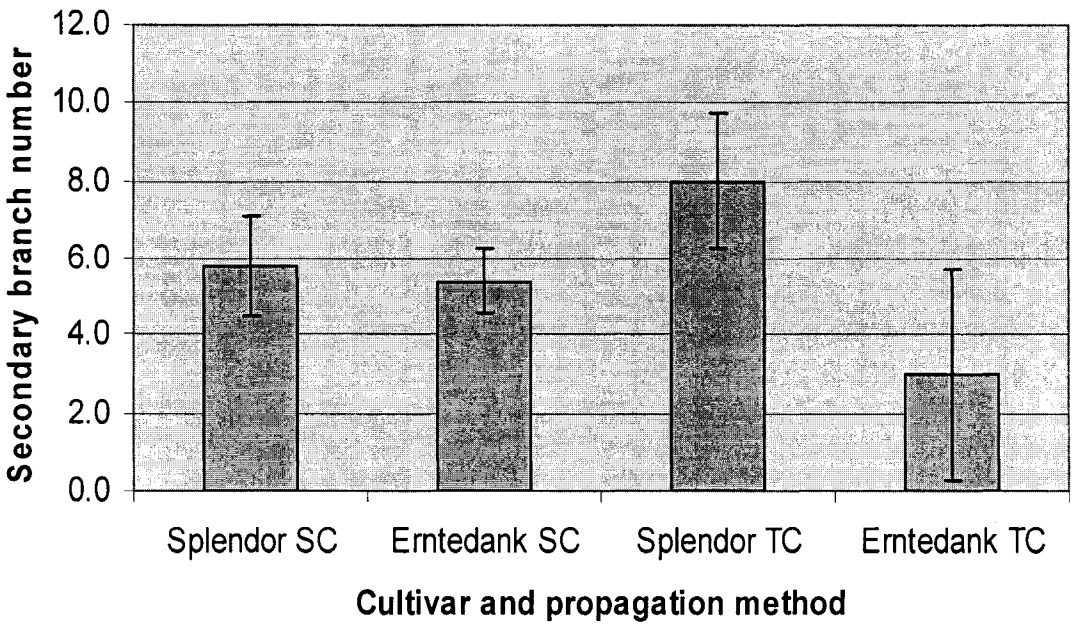

Figure 3.3: Number of secondary branches in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

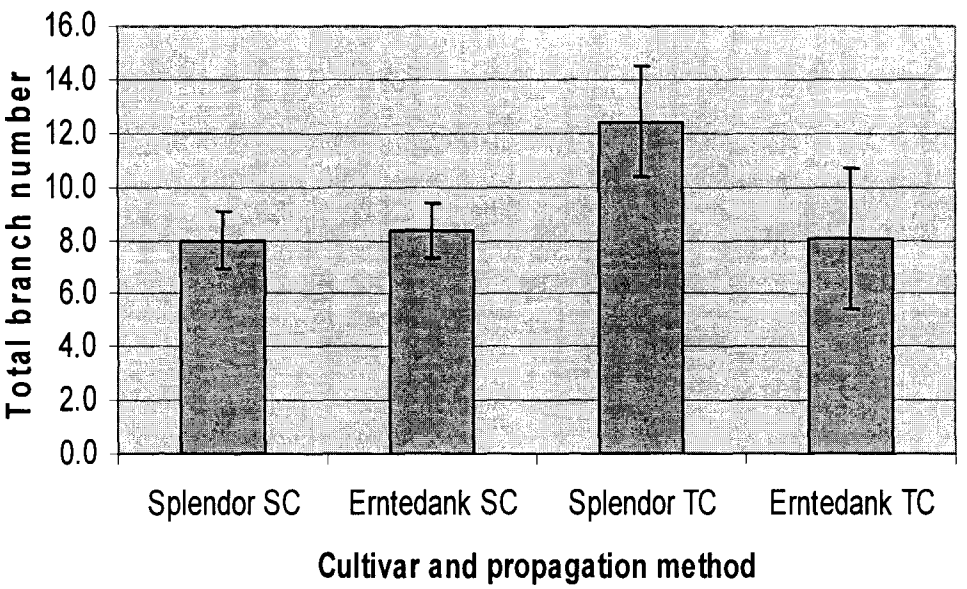

Figure 3.4: Total number of branches in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

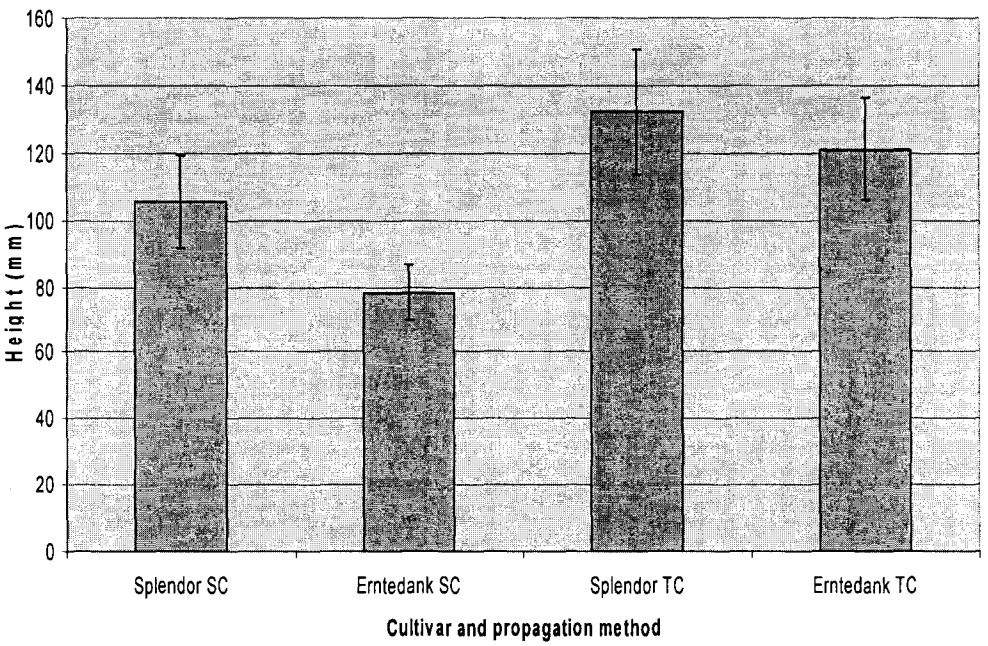

Figure 3.5: Maximum plant height of lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

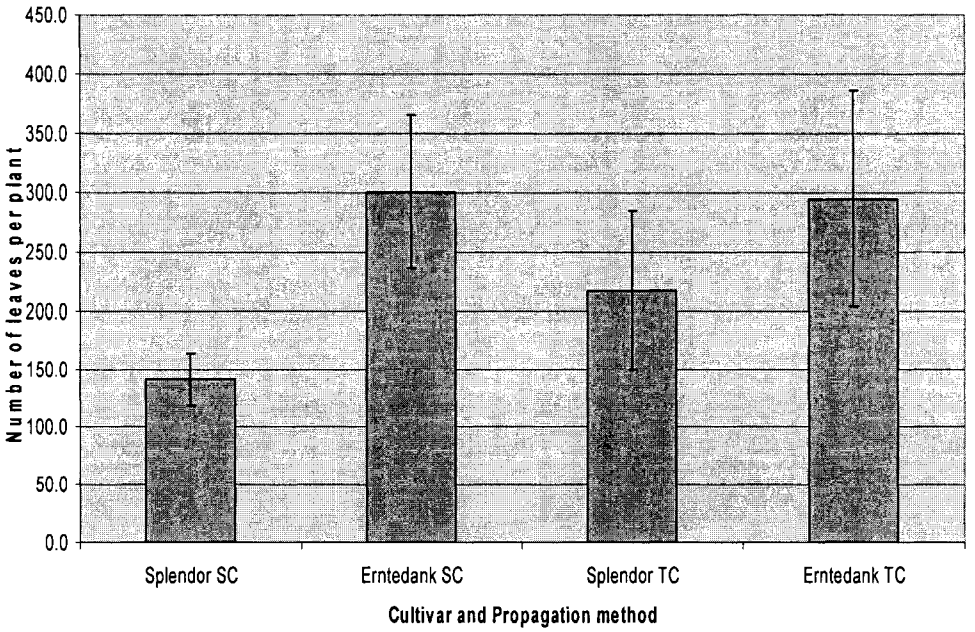

Figure 3.6: Number of leaves per plant in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

3.2 Fruit Characters

After four years of growth both SC and TC plants yielded enough fruit crop to allow analysis of fruit characters and biochemical analysis of the fruit in the same growing season. Significant differences were found between cultivars and between propagation methods for berry diameter, weight and number per plant (Table 2). However interaction between cultivar and propagation method was not important for fruit traits. 'Splendor' plants produced considerably larger fruits than 'Erntedank' (Figure 3.7). 'Erntedank' plants, however, produced a significantly higher number of fruit (Figure 3.8) resulting in an overall greater yield of fruit (Figure 3.9). SC plants produced increased numbers of heavier, larger fruit than plants produced by TC (Table 2.).

Of the two cultivars studied 'Erntedank' plants produce the highest number berries per plant; SC plants produced the highest number of berries between the propagation methods (Table 2). SC plants also produced heavier, larger fruit than TC plants. Although 'Splendor' produced larger fruit, the larger yield in 'Erntedank' resulted in a larger overall fruit yield (g) in the German cultivar (Figure 3.9).

Table 2: Effects of genotype and propagation method (SC = stem cutting, TC = tissue culture) on fruit characters of two lingonberry cultivars measured after four growing seasons.

	Berry diameter (mm)	Berry number per plant	Berry weight per plant (g)
Analysis of variance		P values	
Cultivar (Cv)	<0.001	<0.0001	<0.0001
Propagation method (PM)	0.0181	0.0007	<0.0001
Cv × PM	0.8645	0.0927	0.1001
Means			
Cultivar			
'Splendor'	8.5a	7b	2.4b
'Erntedank'	7.2b	20a	5.3a
Propagation method			
SC	8.0a	17a	5.1a
TC	7.7a	10b	2.6b

Means within columns and factors with different letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test.

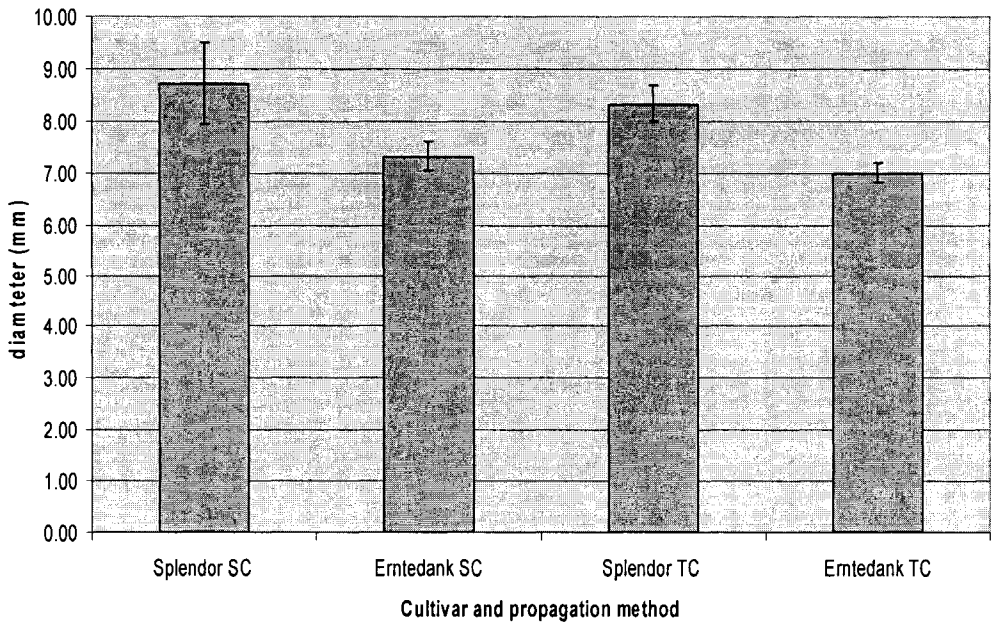

Figure 3.7: Berry diameter in lingonberry cultivars 'Splendor' and 'Erntedank propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error

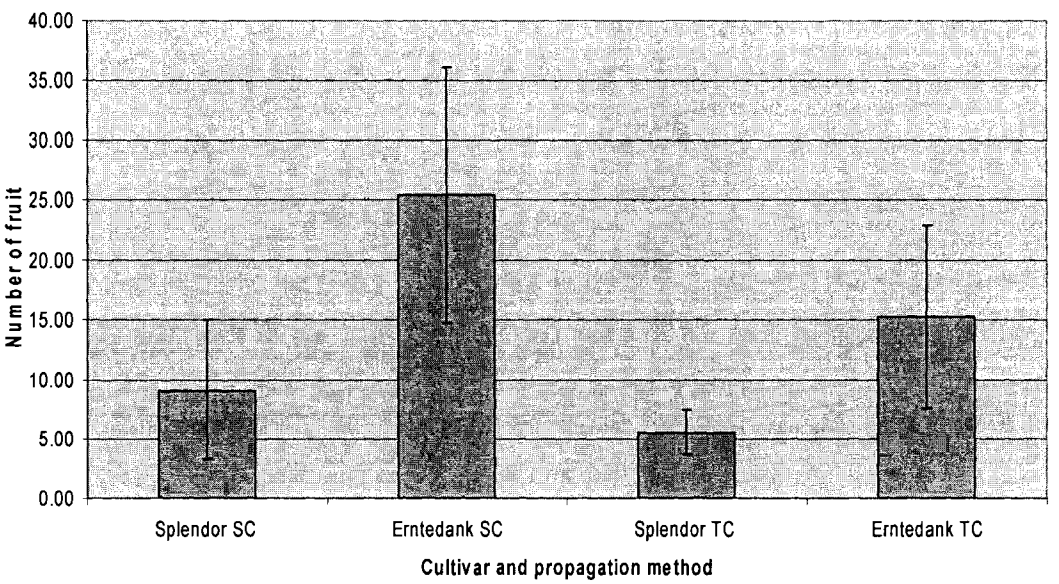

Figure 3.8: Berry number in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

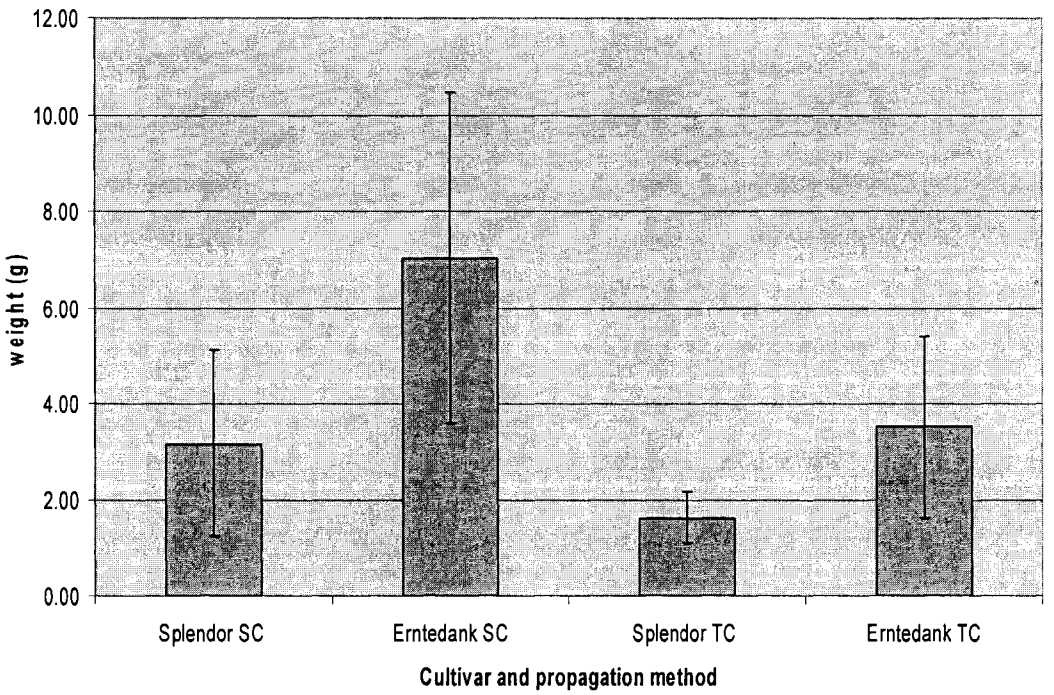

Figure 3.9: Fruit yield (g) in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

3.3 Chlorophyll Estimation

There were no significant differences between cultivars or between propagation methods, for chlorophyll b in the leaves studied, however chlorophyll and total chlorophyll content were significantly different between cultivars (Table 3). No significant difference was found however between propagation methods. The interaction between cultivar and propagation methods was also insignificant (Table 3). The ratio of chlorophyll a/b was 2.33 for the 'Splendor' and 2.28 for 'Erntedank'. The propagation method showed no significant variation with a ratio of 2.33 for TC plants and 2.28 for plants derived by SC plants (data not shown). Figure 3.10 shows a consistent pattern of chlorophyll distribution between cultivars and between propagation methods both within and between cultivars.

	Chlorophyll a (µg per mg)	Chlorophyll b (µg per mg)	Total chlorophyll (µg per mg)
Analysis of Variance		P values	
Cultivar (CV)	0.0364	0.1690	0.0542
Propagation Method (PM)	0.3457	0.1670	0.2273
CV × PM	0.6156	0.4232	0.5328
Means			
Cultivar			
'Splendor'	1.84a	0.94a	2.78a
'Erntedank'	1.67a	0.72a	2.39a
Propagation Method			
SC	2.15a	1.04a	3.19a
TC	2.01a	0.94a	2.95a

Table 3: Effects of cultivar and propagation method (SC = Stem cutting, TC = Tissue culture) on chlorophyll levels of two cultivars of lingonberry

Means within columns and factors with different letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test.

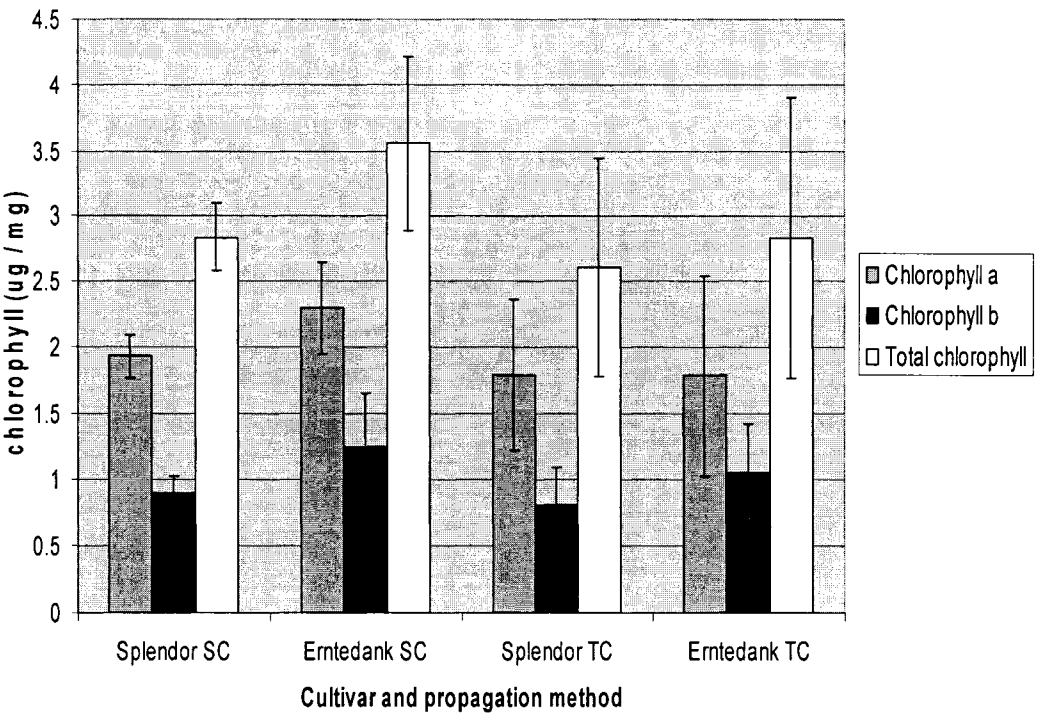

Figure 3.10: Levels of chlorophyll a, chlorophyll b and total chlorophyll in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

3.4 Total Fruit Anthocyanins

Analysis of variance for the combined effect of cultivar and propagation method showed that there was no significant interaction for total anthocyanin level either by the pH differential method or by High Performance Liquid Chromatography method (Table 4). Propagation method, however, significantly affected the level of total anthocyanins ($P = 0.0016$) in the pH differential assay. SC plants possessed berries with higher levels of anthocyanins than those of TC plants in both cultivars when measured by the pH differential method (Figure 3.11). But there was no significant difference for anthocyanins between the cultivars 'Splendor' and 'Erntedank' when measure by HPLC (Table 4).

Table 4: Effects of genotype and propagation method (SC = stem cutting, TC = tissue culture) on total anthocyanin levels and antioxidant activity of two lingonberry cultivars measured after four growing seasons.

	Total anthocyanin by pH differential method (mg /100g)	Total anthocyanin by HPLC (mg /100g)	Antioxidant activity (μmol/g)
Analysis of variance			
		P values	
Cultivar (Cv)	0.4026	0.335	<0.0001
Propagation method (PM)	0.0016	0.2301	<0.0001
Cv × PM	0.7288	0.513	0.0013
Means			
Cultivar			
'Splendor'	106a	134a	135b
'Erntedank'	102a	118a	182a
Propagation method			
SC	115a	136a	148b
TC	94b	116a	163a

Means within columns and factors with different letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test.

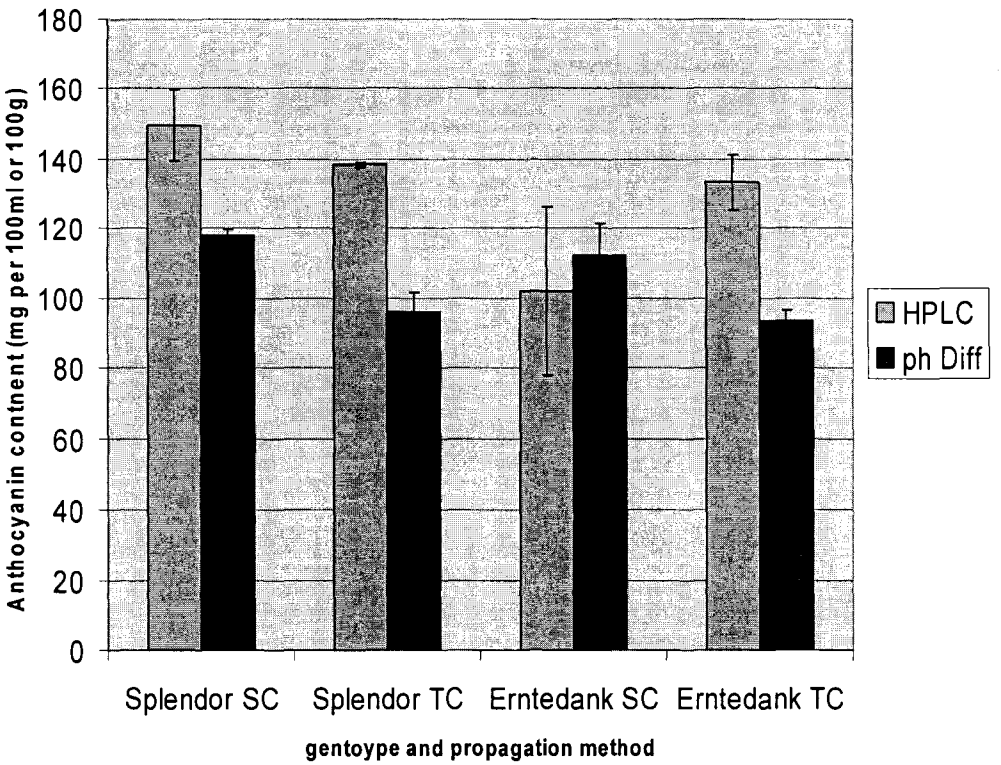

Figure 3.11: Anthocyanin content in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

3.5 High-Performance Liquid Chromatography (HPLC) Separation of Anthocyanins

Analysis of variance for the separation of anthocyanins by HPLC showed no significant difference between cultivars or propagation method (Table 4). The anthocyanin profile of lingonberries consists of three cyanidin analogues with three sugar moieties, cyanidin-3-galactoside (84%), cyanidin-3-glucoside (5%) and cyanidin-3-arabinoside (11%) (Figure 3.12). The results obtained via HPLC show that the levels of the three major anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside and cyanidin-3-arabinoside) do not significantly differ between cultivars or propagation methods, and that there was no interaction between cultivar and propagation method (Table 4). The mean values reported for the levels of anthocyanins quantified from the HPLC chromatographs show that 'Splendor' has means levels of anthocyanins 12% higher than the levels obtained in 'Erntedank'. The means from SC-derived plants were 15% higher than those obtained from TC-derived pants, however, the results were not significant. The chromatographic profile of the two lingonberry cultivars, propagated both by SC and TC, shows the distribution of anthocyanins within the fruit (Figure 3.12).

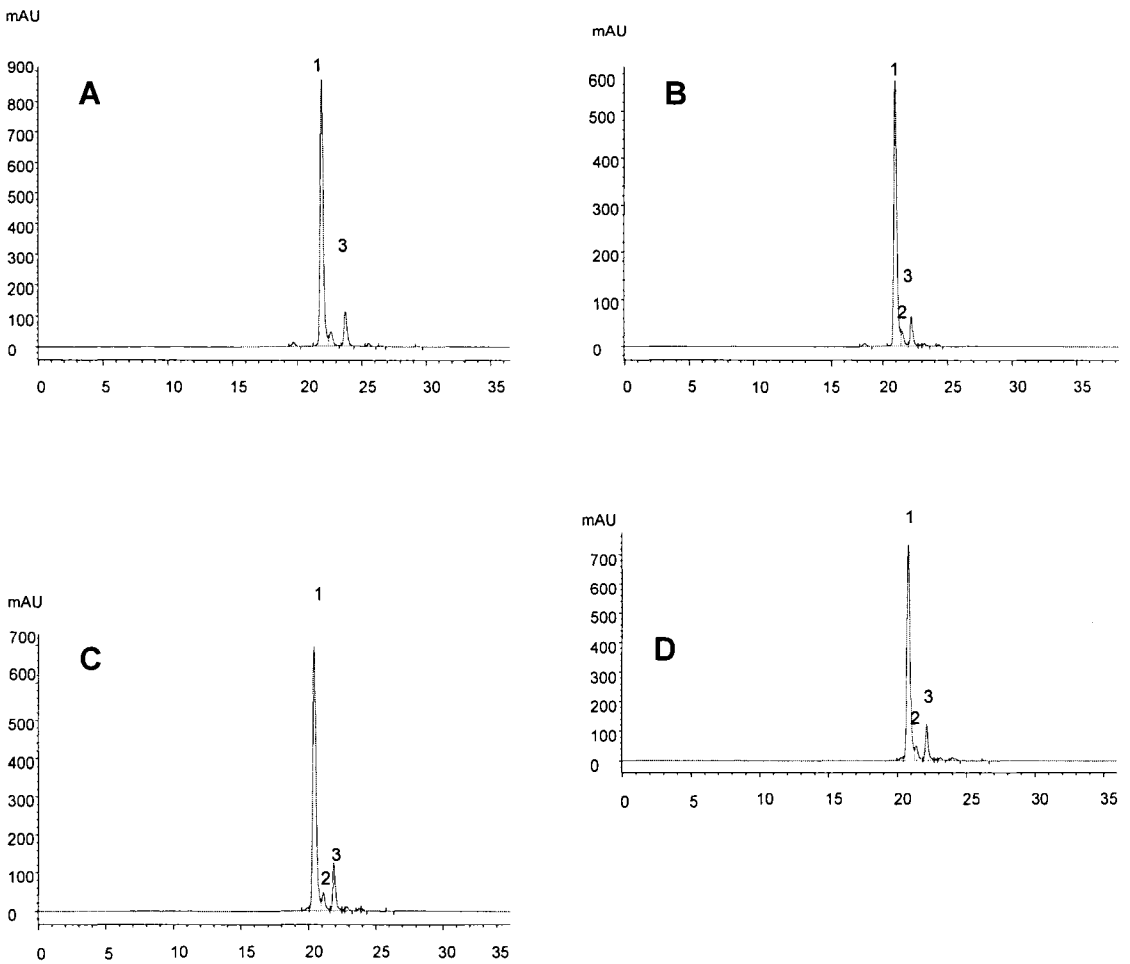

Figure 3.12: HPLC chromatographs of lingonberry fruit extract at 535 nm showing distribution of anthocyanins in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC).
(A = Splendor SC, B = Erntedank SC, C = Splendor TC, D = Erntedank TC).

3.6 Antioxidant Activity

Significant interactions between propagation method and cultivar existed ($p = 0.0013$) for antioxidant activity (Table 4). Cultivars significantly differed in the total level of antioxidant activity, 'Erntedank' fruit had greater activity than did 'Splendor' fruit in both propagation methods (Figure 3.13). The TC plants had a significantly higher level of antioxidant activity in their fruit than did SC plants in both cultivars (Figure 3.13). There is a negative correlation between antioxidant activity and total anthocyanin levels in the present study (data not shown).

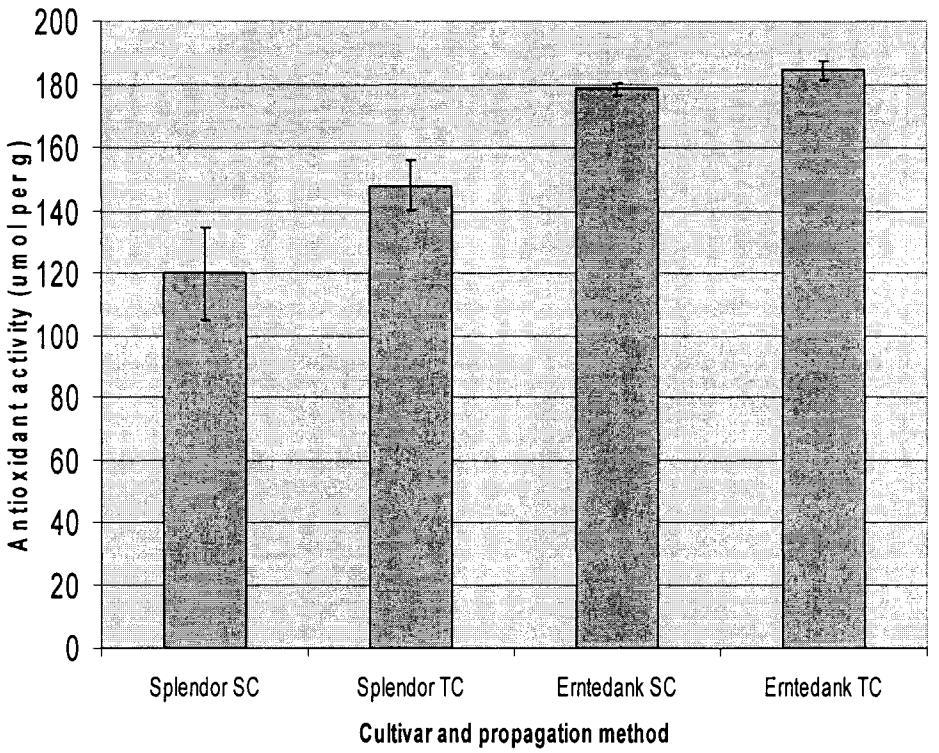

Figure 3.13: Antioxidant activity in lingonberry cultivars 'Splendor' and 'Erntedank' propagated by stem cuttings (SC) and Tissue culture (TC). Error bars indicate standard error.

CHAPTER 4: DISCUSSION

4.1 Morphological Characters

The two cultivars in the present study differed significantly in many important morphological characteristics used to determine the health and fitness of lingonberry plants. The characters in which these plants were significantly different include: stem number, primary, secondary and total branch number, leaf number per plant and plant height. Both cultivars exhibit a high degree of plasticity in adapting to and thriving in the cool and often harsh climate of Newfoundland and Labrador.

The German cultivar 'Erntedank' produced five times the number of primary stems as did the North American cultivar 'Splendor'. The results show that the 'Erntedank' plant produced on average 5.38 stems per plant compared to 1.04 stems per plant for 'Splendor'. In a recent study, 'Erntedank' plants were found to produce 4.9 stems per plant (Debnath 2006); previous to that, 'Splendor' plants were found to have just over 2 shoots per plant (Debnath 2005c). The increased generation of stems is a result of rhizomatous spreading, typically 'Erntedank' plants begin to spread after the third growing season (Penhallegon 2006) and are known to grow densely in natural stands (Zillmer 1985).

Propagation method was also shown to effect the growth and development of plants and their resulting morphologies. Plants propagated by TC showed a far greater stem number than plants propagated by conventional SC methods. Tissue culture plants spread rhizomatously and produced far more rhizomatous stems than do SC plants (Data not shown) as previously reported by Holloway (1985) and Debnath (2006). Tissue culture plants, as a result of prolific stem production, have a larger framework for the development of branches, leaves and rhizomes than SC plants. The increased rhizomatous shoot production of TC plants could be a result of the hormonal stimulus provided initially by the culture medium. Hormones are involved in many plant regulatory functions; presence or absence of certain hormones can inhibit or promote various plant processes. Root formation is part of a polarization of plants and their meristems which relies on auxin to initiate root formation. Auxins are produced in regions nearest to the shoot tip and are carried by a series of membrane-bound transport proteins to the root cap, where auxin accumulates and acts to regulate gene transcription required for root and rhizome production (Wolpert *et al.* 2002). The media used in some TC systems have auxins added to initiate rooting *in vitro*, however, this initial excess of auxin could initiate accelerated rhizome production that results in the impressive vegetative growth and dense canopy formation commonly associated with TC plants. The medium used in the present experiments contains a cytokinin (zeatin), a hormone that initiates root and stem development. ANOVA showed also a significant cultivar-based effect on the production of primary, secondary and total number of branches (Table 1). The 'Erntedank' cultivar produced nearly double the number of primary branches, two

more secondary branches and 62% more total branches per plant than did the 'Splendor' cultivar. On average 'Erntedank' plants had 4.08, 7.72 and 12.40 primary, secondary and total branches respectively compared to 2.13, 5.67 and 7.73 for 'Splendor'.

Increased branching or some other morphological adaptation can be an appropriate plastic response to environmental variation (Donohue *et al.* 2001). These plants were all greenhouse maintained in 4 inch pots for 4 years, grown side by side, in plastic trays each containing 15 pots, densely grouped together and all competing for favourable environmental stimuli. The potting medium in the pots is a 60 : 40 ratio of peat : pearlite respectively. The potting medium was not replaced during the study, soil nutrients were subsidized using 20-20-20 NPK (nitrogen-phosoporous-potassium) fertlizer. Increased branching in the plants studied could possibly be an adaptive response to the crowded conditions to which the plants were placed, over time the root and rhizome mass below the soil increased and the pots acted as a limiting factor on the spreading of the plants. When propagation methods are compared they show significant variation in their branching patterns and distribution of branching. TC-derived plants show increased primary branching, however TC plants show very little in the way of secondary or tertiary branching. SC plants are well branched, and show many secondary and therefore total branches. The branching of SC plants is important, as they are limited to producing only one primary stem. Increased branching leads to increased surface area, which provides more area for leaf and flower attachment. This increase in branching helps plants compete against nearby plants for environmental stimuli such as light, water and wind.

4.2 Fruit Characters

Fruit production, like the morphology showed significantly high levels of cultivar-based variation. 'Splendor' plants produced one third the number of fruit of 'Erntedank' plants in the present study. However, the average berry diameter and the individual berry weight were significantly higher in 'Splendor' plants. Penhallegon (2006) described similar trends in fruit size, although he described both plants as being high yielding after three years. The results in Table 2 show the mean values for berry diameter for 'Splendor' and 'Erntedank', respectively, were 8.5 mm and 7.2 mm, the average weight of fruit produced per plant was 2.4 g and 5.3 g for 'Splendor' and 'Erntedank' respectively. The number of fruit harvested per plant was 20 in 'Erntedank' and 7 in 'Splendor'.

Although the reason for this cultivar-based difference is not known, several possibilities exist. It is possible that the large fruit produced by 'Splendor' plants act to attract foraging animals to consume the fruit allowing them to use animal vectors to transport the seed away from the original plant. Large fruit size is a very important basis for the selection of 'Splendor' plants, as increased fruit size is a pre-existing variation found in this cultivar. Conversely, 'Erntedank' plants produce high yields of smaller fruit. This reproductive strategy has its own distinct advantages as well. The German variety,

by producing smaller fruit required less energy from metabolism, water and sugars to produce ripe fruit and can produce more fruit per plant.

The propagation method also significantly affected fruit characters in the two cultivars studied. Although statistically insignificant for berry diameter, propagation method affected the yield of the plants, as well as the weight of the fruit produced. SC plants produced an average of seven more fruit per plant than TC plants (Table 2), the net berry weight per plant in SC was nearly double that of TC plants. Previous studies indicate that micropropagated blueberry plants consistently out-produced conventionally produced blueberry plants in shoot production and fruit yield (Smagula and Lyrene 1984). Similar results were obtained in lingonberry (Gustavsson 1997). The improved fruit yields were due to the high numbers of branches formed in the initial vegetative growth phase, giving more surface area for flower and fruit production (Debnath 2005b). The results of the present study contrast with these results, indicating SC plants out yielded TC plants for fruit. However, TC out-produced SC plants for shoot number. The increased vegetative production of TC plants provides an interesting situation with regards to nutrient availability in the pots. With so many stems, branches and leaves there is a greater nutrient requirement than SC plants. However, all pots contained the same volume of potting medium, as well, they receive the same irrigation and fertilizer schedule. The result is a differential nutrient availability per stem that could affect the fruit yield of the plants due to nutritional restrictions places on TC-derived plants, whereas the SC-derived plants receive enough nutrients to produce as much fruit as possible.

Plants produced by TC direct significant amounts of energy into the production of new axillary shoots and rhizomes and so are potentially bound to vegetative production that restricts the number of fruit and the maximum size of the fruit produced. The increased vegetative growth and dense rhizome networks of TC plants are energetically expensive on the plant. The production of so many shoots and rhizomes requires energy that would otherwise be used in flower and subsequently fruit production. Although given sufficient time and nutrient availability, fruit production may be increased and even greater than SC plants due to the number of stems TC plants produce. TC plants also produce a greater number of leaves per plant than those of SC plants. Leaves produce energy by photosynthesis; this energy in turn can be used for fruit production. In contrast, the results suggest that SC plants show energy conservation by producing few if any rhizomes and one primary shoot, allowing for greater flower production that leads to increased fruit yield.

4.3 Chlorophyll Estimation

Chlorophyll, the green pigment common to all photosynthetic cells, absorbs all wavelengths of visible light except green. The measure of chlorophyll is a very important parameter in plant physiology. The ratio of chlorophyll a/b is indicative of photosynthetic activity as well as the health of the plant (Johnston *et al.* 1984).

In the present study, we found that cultivar and propagation methods had no significant effect on the synthesis of chlorophyll b, however chlorophyll a and total

chlorophyll content were significantly affected by cultivar and not the propagation method (Table 3). Plants from both cultivars produced nearly equal amounts of chlorophyll a, indicating that each variety could absorb light equally well in the violet-blue and orange-red wavelengths. Chlorophyll a synthesis was greater in 'Splendor' plants, which yielded 1.84 μg per mg compared to 1.67 μg per mg in 'Erntedank'. Chlorophyll b synthesis was nearly equal in both varieties, showing that light energy absorption was equal in the green wavelength as well as the orange-red range on the spectrum of visible light. Chlorophyll b is an accessory pigment that acts indirectly in photosynthesis by transferring the light it absorbs to chlorophyll *a*, it absorb energy from wavelengths that chlorophyll a does not absorb.

The results for all plants in this study indicated a low chlorophyll ratio (data not shown). There are previously-published reports that give alternate possible explanations for reduced chlorophyll ratios. The normal expected chlorophyll a/b ratio in vascular plants is 3.0 (Porra 2002). Bryophytes that are adapted to living in shady conditions generally have reduced chlorophyll a/b ratios ranging from 1.94 to 3.33 (Marschall and Proctor 2004). Angiosperms in low light conditions have ratios of 2.12 to 3.29 with a mean value of 2.74 in light acclimation experiments (Johnson *et al.* 1993). Under periods of low light, the content of the accessory pigment chlorophyll b increases to maximize light energy absorption. The increase in chlorophyll b content, together with no net change in chlorophyll a content reduces the chlorophyll a/b ratio. The plants in the present study are so crowded that they provide shade to one another throughout the day. Shade is also provided by other nearby plants and by structural elements in the

greenhouse. One study found that nitrogen deficient plants had increased stress responses including reduced chlorophyll levels (Bongue-Bartelsman and Phillips 1995). Another study found that sodium-deficient C_4 plants had significantly reduced chlorophyll a/b ratios (Johnson *et al.* 1984), while another study suggests that chlorophyll b synthesis increases during times of plant stress, and by doing so reduced the chlorophyll a/b ratio (Lahai *et al.* 2003).

4.4 Total Fruit Anthocyanins

The pH differential method is a fast, accurate and cost-effective procedure for the analysis of total anthocyanins from fruit extracts (Jones *et al.* 2003). The results indicate that there was no difference in total anthocyanin level between cultivars (Table 4). 'Splendor' and 'Erntedank' fruits contained 106 and 102 mg of total anthocyanins per 100 g frozen fruit respectively. Although there is a lot of variation between cultivars for anthocyanin levels in lingonberry, 'Splendor' and 'Erntedank' have been reported to contain similar levels of anthocyanin (Wang *et al.* 2005). The values obtained in the present experiment are higher than the values obtained by Wang *et al.* (2005). SC plants produced higher levels of anthocyanins in the fruit than did TC plants by approximately 18%. SC plants produced 115 mg of total anthocyanins per 100 g frozen fruit compared to 94 mg of total anthocyanins per 100 g frozen fruit from TC plants. The use of TC methods appears to affect the chemical composition of the fruit of the plants produced, and decreases the anthocyanin content compared to SC methods. Several other

possibilities exist however that could explain the difference in anthocyanin levels, one factor to consider is temperature.

Temperature is known to affect the accumulation of anthocyanins in fruit skins (Spayd *et al.* 2002). The cool climate of Newfoundland and Labrador could play a part in the elevated synthesis of anthocyanins in the lingonberry fruit. It was found that ripening under low temperatures produced higher levels of anthocyanin in fruit in four *Berberis* (barberry) species (Laleh *et al.* 2006). Similar results were also found with grape berry skins (Yamane *et al.* 2006). One possible explanation for the increased anthocyanin content in Newfoundland and Labrador produced fruit over previous studies could be in the regional temperature difference between the study sites. The normal annual temperature for the St. John's based ACCCRC in 2004 was 6.98° C with a maximum of 24.51° C in August and a low of -0.02 ° C in January (Environment Canada 2005). The paper published by Wang *et al.* (2005) used lingonberry fruit collected in Fall Creek, Walker and Whitham Oregon. The average temperature in Oregon annually is 17.3 ° C, with a max of 29.32 ° C in July and a low of 2.63 ° C in January. The data from the pH differential method experiment together with the mean temperature values indicate that there might be a correlation between decreased environmental temperature and increased levels of anthocyanin production in lingonberry fruit.

4.5 High-Performance Liquid Chromatography (HPLC) Separation of Anthocyanins

The distribution and composition of anthocyanins in fruit play a role in the level of therapeutic effects they provide (Wang and Jiao 2000, Rossi *et al.* 2003). Anthocyanin levels in fruit are affected by many factors including analytical methods, genotype, growing conditions and stress factors (Macheix 1990). There was no significant variation between cultivars or propagation method for total anthocyanins using HPLC (Table 4). Despite the mean values being separated by nearly 20 mg per 100 g frozen weight, standard error caused the values for cultivars to be statistically insignificant. 'Splendor' and 'Erntedank' fruit contained 134 mg and 118 mg of anthocyanins per 100 g frozen fruit, respectively. The results for total anthocyanins by HPLC confirm the results from the pH differential method. The values obtained show a similar trend, but are higher than those by Wang *et al.* (2005). Wang *et al.* (2005) reported the average levels of anthocyanins found in berries of several common lingonberry cultivars harvested in August and October. There are insignificant levels of variation in total anthocyanin levels between the 'Splendor' and 'Erntedank' cultivars. The chromatographic profile resulting from separation of anthocyanins (Figure 2.9) closely resembled those obtained by Andersen (1985) and Kahkonen *et al.* (2003). No significant differences were observed in anthocyanin content between SC plants and TC plants (Table 4). Although SC plants had higher average anthocyanin content than TC plants, the mean values agreed with those

obtained in the pH differential method. The SC fruits contained 136 mg anthocyanins per 100 g frozen fruit while TC fruits had 116 mg anthocyanins per 100 g frozen fruit.

Plant stress is very influential on the growth and developmental patterns in plants (Gusta *et al.* 2005). There are several types of plant stress including: drought, heat, mineral deficiency / toxicity, salinity, oxidative stress, water logging, handling stresses and cold (Gusta *et al.* 2005). The plants studied were grown and maintained in small four-inch pots over a period of four years. All plants received the same irrigation and fertilizer application despite TC plants having more stems, leaves and denser canopies (Table 1) and increased rhizome networks (Debnath 2006). Plants with denser canopies and increased shoot numbers have higher nutritional requirements than plants with fewer shoots and leaves. Plants with more shoots, leaves, rhizomes and roots experience drought-like stress by having to divide the available nutrients throughout the plant. Space restrictions based on small pot size also limit the available micronutrients from the potting medium, which are depleted over time causing nutritional stress on the plants. Plant stresses possibly play a role in the growth patterns and developmental biology of the plants in the present study and possibly act in an additive manner resulting in increased levels of anthocyanins as a stress response. The accumulation of anthocyanins in plants is often a result of some environmental stimulus (Yamane *et al.* 2006) and varies seasonally (Sivac and Sokmen 2004). The accumulation of anthocyanins occurs for a variety of reasons, it is a natural developmental process however stress, environmental response and human interaction are several other potential causes.

Anthocyanin accumulation commonly occurs in young expanding leaves, it also commonly occurs in plants experiencing macronutrient deficiencies often caused by agricultural production where nutrients from the soil are removed and replaced with fertilizer (Close and Beadle 2003). Because at this developmental stage the leaves are not yet photostable, young expanding leaves experience an accumulation of anthocyanins, which is produced gradually during leaf expansion (Choinski and Wise 1999). High anthocyanin levels in young developing leaves have previously been correlated with delayed or gradual chlorophyll synthesis (Drumm-Herrel and Mohr 1985; Dodd *et al.* 1998). Until leaves are fully photostable they are not able to dissipate excess light energy and are vulnerable to photodamage (Close and Beadle 2003). Foliar anthocyanins accumulate as a result of nitrogen (N) deficiency in many plant species (Close *et al.* 2000). Nitrogen deficiency affects anthocyanin gene expression, this results in increase production of anthocyanins in affected plants (Bongue-Bartelsman and Phillips 1995).

4.6 Antioxidant Activity

Currently there are a number of procedures available for analysis of antioxidant activity; however, there is no accepted standard evaluation technique (Frankel and Meyer 2000). In the present study the ABTS method was performed. This technique is accurate, low cost and not labour intensive, which makes it a good candidate for use in fruit analysis (Hanson *et al.* 2004).

The cultivars in the present study differed significantly in antioxidant activity; 'Erntedank' fruit had higher activity than did those of 'Splendor'. These results agree with Wang *et al.* (2005) who surveyed the antioxidant capacity in several lingonberry cultivars. Wang *et al.* (2005) found that ripe 'Erntedank' fruit had greater antioxidant activity than 'Splendor' using the oxygen radical absorbance capacity (ORAC) assay. The cultivar-based difference in antioxidant activity could be due to the elevated presence of phenolics. Wang *et al.* (2005) reported that ripe 'Erntedank' fruit harvested in August had 719.2 ± 18.1 mg per 100 g of total phenolics, while ripe 'Splendor' fruit had only 549.3 ± 6.7 mg per 100 g of total phenolics. This significant difference in phenolic content could account for the cultivar-based difference in antioxidant activity as phenolics are known to have potent antioxidant activities (Beta *et al.* 2005).

Propagation method had a significant effect on antioxidant activity. TC plants produced fruit with higher activity to SC-produced fruit (Table 4). There are no previous reports regarding the effect of propagation method on antioxidant activity in lingonberries.

The results of the present antioxidant activity study negatively correlate with the anthocyanin content analysis by the pH differential method and contradict the reports of Wang *et al.* (2005) who found a positively correlation of anthocyanin levels with antioxidant activity. However, this is the first report of the effect of propagation method on the antioxidant level or anthocyanin level in TC plants compared to SC plants. Plants from the same cultivar react differentially to SC and TC plant propagation techniques.

The differences obtained in the present study can be at least partially attributed to the different propagation methods and conditions of propagation used in the preparation of the plant population. The occurrence of somaclonal variation is another possible explanation for the results observed in the present set of experiments.

Variation in plants regenerated from tissue culture is defined as somaclonal variation (Lee and Phillips 1988). Somaclonal variation is heritable; they are passed on through meiosis and for the most part are irreversible (Tremblay *et al.* 1999). Although unlikely to occur in all plants studied, this variation in isolated cases can result in significant changes in phenotype and biochemistry of the plants (Trembley *et al.* 1999). During the tissue re-differentiation phase of TC adventitious shoot regenerations it is likely that somaclonal variation could alter gene activity and create novel and beneficial changes in antioxidant activity. In studies on several important horticultural crops including sunflower, chilli pepper and geranium; somaclones were found to be more desirable than the explant source (Trader *et al.* 2006).

Significant chromosomal rearrangements have been documented in plants treated *in vitro* (Harris 1964). The basis for this chromosomal rearrangement is a difficult process to understand, several possible causes are plausible. Late replicating heterochromatin is one possible explanation for the chromosomal aberrations expressed in TC plants (Lee and Phillips 1988). Chromatin, a cell's DNA and its associated protein factors, is found in two varieties: euchromatin and heterochromatin. Heterochromatin or areas of a chromosome that are genetically inactive because they either lack genes or contain genes that are repressed replicates later in S phase of the cell. Both centromeres

and telomeres are heterochromatic, as well as the Barr body of the second inactivated X chromosome in a female. Heterochromatin is usually localized to the periphery of the nucleus (Bryant 1976). The involvement of late-replicating heterochromatin in the generation of somaclonal variation is not isolated to tissue culture plants, but is also documented in Drosophila (Halfer *et al.* 1980), cattle (Vig 1982) and humans (Stalder *et al.* 1965). Transposable-element activity is another likely source of somaclonal variation in TC-derived plants (Larkin and Snowcroft 1981). Transposable elements have common effects on plants such as frequent reversions of unstable alleles and the production of single gene variants (Lee and Phillips 1988). The instances of transposable elements *in vitro* are increased compared to *in vivo* systems (Rubin 1983). One locus where reversion of unstable alleles and recovery of new alleles has been documented in regenerated plants is the anthocyanin pigmentation locus (Lee and Phillips 1988, Groose and Bingham 1986).

It should be noted that there are epigenetic sources of variation that are heritable, but reversible that could be responsible for the observed results (Russo *et al.* 1996). There are methods to determine whether somaclonal variation or epigenesis has occurred. Analyses of phenotype, chromosome number or direct DNA evaluation are several of them (De Klerk 1990). Some studies have shown that there is pre-existing variation in the explant source that is expressed within regenerated plants (D'Amato 1986). Also, the culture regime used, as well as the composition of the media (hormones) have been shown to affect the cytological status of cultured cells (Torrey 1961, Harris 1964), the level and extent of chromosomal variation increases with the duration of *in vitro* growth

(Lee and Phillips 1988). Others believe that the culture medium is mutagenic to plants and causes polymorphisms within the plant cell causing morphological, biochemical or genetic changes (Bayliss 1973). There is also evidence that chromosomal aberrations within *in vitro* generated cells results in somaclonal variation (Lee and Phillips 1988).

CHAPTER 5: CONCLUSIONS

While little is known presently about the long term effects of *in vitro* tissue culture effects on lingonberry, the results of this thesis suggest TC systems produce horticulturally-superior plants than conventional SC systems. However, TC plants are adversely affected for fruit yield and anthocyanin levels while having superior antioxidant activity after four years of growth under greenhouse conditions.

The affinity of lingonberries for Newfoundland and Labrador climate and soil conditions make this crop a promising candidate for intense horticultural development. This plant has economic significance as it is in high demand in European countries as well as domestic and local markets. Lingonberries are a nutritional fruit that have many uses. Already in agricultural production in Europe and various locations in North America, it is my belief based on the results obtained here that lingonberry should be introduced in Newfoundland as a sustainable small fruit crop. The use of micropropagation techniques such as adventitious shoot regeneration from excised leaves consistently produced superior plants to conventional softwood stem cuttings for many agriculturally important characters. Modern techniques such as tissue culture of leaf explants are fast, infection-free methods for rapid establishment of huge numbers of

plants. When used synergistically with conventional methods these tissue culture techniques can maintain germplasm stocks while producing superior plants. Appropriate cultivar selection, combined with Newfoundland and Labrador's climate and soil conditions could result in prolific production of lingonberries that would complement the existing wild supply of fruit. Research at the Agriculture and Agri-Foods Canada Atlantic Cool Climate Crop Research Center is at the forefront of such research. Programmes such as the sustainable crops initiative, small fruit programme, and biotechnology branch of research focus on the agricultural needs for future in Newfoundland and Labrador.

In conclusion, this study showed that cultivar and propagation method significantly influence the growth and development of lingonberries. Adventitious shoot regeneration from excised leaves results in lingonberries having desirable morphological characters as seen in increased shoot, leaf and rhizome production. Conventional propagation methods showed increased fruit production and yield despite reduced vegetative production. The development of an improved lingonberry programme would best be served by using micropropagation together with conventional softwood cuttings and plant breeding to consistently produce fruit of the highest quality in large numbers. Development of new cultivars with greater yields and health benefits should be given high priority in Newfoundland and Labrador.

Long term studies on the effects of *in vitro* propagation systems on lingonberry are required to determine the effects of propagation on fruit yields and chemical composition as well as the fitness of the plants after transfer to the soil. Genetic analyses are important in determining the source of the advantageous changes brought on by TC

techniques, and whether they are heritable or epigenetic. Breeding programmes that cross European and native Newfoundland and Labrador plants should be implemented to develop better cultivars for production. Networking between governments, industry and university is fundamental in establishing research partnerships in agriculture that would benefit the local farmers and province of Newfoundland and Labrador.

In loving memory of

Daniel B. Foley

1959 – 2003

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